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Report Number AN-2-1987

FUNCTIONAL CONSEQUENCES OF CHEMICAL MODIFICATION OF THE SAXITOXIN BINDING SITE ON NEURONAL SODIUM CHANNELS.

Annual Summary Report
Period 9/1/86 through 8/31/87

Bruce K. Krueger, Ph.D.

December 15, 1987

Supported by:

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MARYLAND 21701-5012

Contract No. DAMD17-85-C-5283

University of Maryland School of Medicine
660 West Redwood Street, Baltimore, Maryland 21201

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REPORT DOCUMENTATION PAGE

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1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) AN-2-1987	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of Maryland at Baltimore, Sch. of Medicine 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) 660 W. Redwood St. Baltimore, MD 21201	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Comm. 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-85-C-5283
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 61102A; PROJECT NO. 3M1-61102BS12; TASK NO. AA; WORK UNIT ACCESSION NO. 121

11. TITLE (Include Security Classification)
Functional Consequences of Chemical Modification of the Saxitoxin Binding Site on Neuronal Sodium Channels.

12. PERSONAL AUTHOR(S)
Bruce K. Krueger, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 9/1/86 TO 8/31/87	1987 December 15	21

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Membranes, ion channels, single channels sodium, saxitoxin batrachotoxin, neurotoxins, Gonyalux, sea anemone toxin, veratridine, rate theory modeling
06	16		
06	20		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Sodium channels from rat brain have been studied at the single channel level in planar phospholipid bilayer membranes in the presence of the activating neurotoxins, batrachotoxin (BTX) and veratridine (VER). Three problems have been investigated. First, an Eyring rate theory model has been developed to account for ion movement through the channel under a variety of experimental conditions assuming that permeant and blocking cations interact with the toxin binding site as they enter the channel. In addition to satisfactorily describing the movement of sodium ions through the channels and block by calcium ions, the model predicted that the marginally permeable potassium ion should be a voltage-dependent blocker. This prediction has now been verified experimentally. In order to examine the generality of voltage-dependent toxin block, we have begun to study some of the properties of veratridine (VER) activated sodium channels. Although voltage-dependent gating and voltage-dependent block by STX and TTX were similar in VER and BTX-activated channels, there appeared to be two distinct VER-activated channel types but only one BTX-activated channel.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia M. Miller	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

SUMMARY

Sodium channels from rat brain have been studied at the single channel level in planar phospholipid bilayer membranes in the presence of the activating neurotoxins, batrachotoxin (BTX) and veratridine (VER). Three problems have been investigated. First, an Eyring rate theory model has been developed to account for ion movement through the channel under a variety of experimental conditions assuming that permeant and blocking cations interact with the toxin binding site as they enter the channel. In addition to satisfactorily describing the movement of sodium ions through the channels and block by calcium ions, the model predicted that the marginally-permeable potassium ion should be a voltage-dependent blocker. This prediction has now been verified experimentally. In order to examine the generality of voltage-dependent toxin block, we have begun to study some of the properties of veratridine (VER) activated sodium channels. Although voltage-dependent gating and voltage-dependent block by STX and TTX were similar in VER and BTX-activated channels, there appeared to be two distinct VER-activated channel types but only one BTX-activated channel. The single channel conductances of VER-activated channels were only about 5 and 10 pS as compared to 25 pS for BTX-activated channels under the same ionic conditions. Biochemical experiments indicated that BTX and VER-activated Na channels can be purified from detergent extracts of rat brain and reconstituted in phospholipid vesicles. The purified channels can then be incorporated into planar bilayers for characterization. With this procedure, the two types of VER-activated Na channels were separated and were revealed to have slightly different molecular weights (about 235,000 and 265,000 corresponding to the 10 pS and 5 pS channels when reconstituted into planar lipid bilayers). The development of STX/TTX-sensitivity of Na channels has been studied in primary cultures of neonatal rat brain astrocytes (glial cells). Initially, the cells has toxin-insensitive (K_i > 100 nM) Na channels as revealed by BTX-activated ^{22}Na influx. High toxin sensitivity appears from day 7 - 10 in culture, probably by insertion of newly synthesized toxin-sensitive channels.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. NIH 85-23, Revised 1985).

Animals are maintained in the Central Animal Facility of the University of Maryland School of Medicine. Animals are housed, cared for, and used strictly in accordance with USDA regulations. The University of Maryland School of Medicine Central Animal Facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. The program of animal care is directed by a full-time, specialty trained, laboratory animal veterinarian. This institution has an Animal Welfare Assurance on file with the NIH Office for Protection from Research Risks (OPRR), Assurance Number A0472.

The person responsible for the Central Animal Facility is:

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Medical School Teaching Facility, Redwood and Pine Streets
Baltimore, Maryland 21201 Phone (301) 528 7612

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EXPERIMENTAL RESULTS

A. Specific Aims of Original Proposal.

The original specific aims (listed below) have not changed. During the first year, substantial progress was made on aims 1, 2, and 4 (see report AN-1-1986). Progress during the present reporting year has been made on specific aims 4 and 5. In addition, work has expanded on aim 1 in new area related to the development of STX and TTX binding and block in brain glial cells in culture (section E).

1. to determine the molecular basis of the voltage dependence of saxitoxin (STX) and tetrodotoxin (TTX) block of neuronal sodium channels.

2. to examine the effects of trimethyloxonium (TMO; a modifier of the negatively-charged toxin binding site) on ion permeation through the channels and on channel block by calcium and strontium.

3. to examine the effect of other carboxyl modifying reagents on ion permeation and block. Special attention will be paid to carbodiimides which render sodium channels insensitive to TTX.

4. to utilize the information in 1 - 3 above to derive a rate theory model for ion permeation through the channel.

5. to determine the rates of opening and closing of single sodium channels at varying membrane potentials and the effects of TMO treatment on these processes.

B. Publications and Scientific Meetings.

Publications: (The first two items were listed in the first annual report [AN-1-1986] but appeared in print during the present reporting year.)

Krueger, B. K., J. F. Worley III, and R. J. French. 1986. Block of sodium channels in planar lipid bilayers by calcium and guanidinium toxins. Are the mechanisms of voltage dependence the same? Annals of the New York Academy of Sciences. 479: 257-268.

French, R. J., J. F. Worley, W. F. Wonderlin, and B. K. Krueger. 1986. Three sites of calcium block in single sodium channels? Proceedings. of the Eighth Annual Conference of the IEEE Engineering in Medicine and Biology Society. 962-965.

Worley, J. F., R. J. French, and B. K. Krueger. Effects of divalent cations and membrane surface charge on ion permeation through single sodium channels from rat brain incorporated into planar lipid bilayers. Submitted to Journal of General Physiology.

Worley, J. F., W. F. Wonderlin, B. K. Krueger, and R. J. French. Ion permeation, divalent ion block and chemical modification of single sodium channels. Description by a 4-barrier, single occupancy, rate theory model. Submitted to Journal of General Physiology.

Cukierman, S., W. C. Zinkand, R. J. French and B. K. Krueger. Effects of membrane surface charge and calcium on the gating of rat brain sodium channels in planar bilayers. in preparation.

Wonderlin, W. F., French, R. J. and N. J. Arispe. Recording and analysis of currents from single ion channels. in preparation.

Abstracts:

Corbett, A. M., W. C. Zinkand, and B. K. Krueger. 1986. Saxitoxin (STX) and calcium interactions with purified sodium channels reconstituted in planar lipid bilayers. Society for Neuroscience Abstracts 12: 45.

Yarowsky, P. J., R. Johnson, W. C. Zinkand and B. K. Krueger. 1987. Development of sodium channels in rat cerebral astrocytes. 1987. Federation Proceedings 46: 504.

Corbett, A. C., W. C. Zinkand, and B. K. Krueger. 1987. Activation of single neuronal sodium channels by veratridine and polypeptide neurotoxin in planar lipid bilayers. Biophysical Journal 51: 435a.

Corbett, A. M. and B. K. Krueger. 1988. Purification and characterization of two sodium channels from rat brain with different alpha subunits and distinct functional properties. Biophysical Journal. submitted.

Cukierman, S., W. C. Zinkand, R. J. French, and B. K. Krueger. 1988. Effects of calcium and lipid surface charge on sodium channel gating. Biophysical Journal. submitted.

Scientific Meetings.

R. J. French presented a paper at the Eighth Annual Conference of the IEEE/Engineering in Medicine and Biology Society, Fort Worth, TX, 11/86. (B. K. Krueger, co-author)

B. K. Krueger, W. C. Zinkand and A. M. Corbett presented a paper at the Biophysical Society meeting, New Orleans, LA, 2/87.

B. K. Krueger, W. C. Zinkand and P. J. Yarowsky presented a paper at the FABEB meeting, Washington, D.C., 3/87.

C. Rate Theory Modeling.

Methods. All experiments described in this section have been carried out using membrane vesicles prepared from homogenates of rat brain that are enriched in ^{3}H-STX binding sites (1). Sodium channels were incorporated into phosphatidylethanolamine-phosphatidylserine planar bilayers and studied in the presence of the activating toxin, batrachotoxin (BTX; 2,3). We have described ion movement through BTX-activated sodium channels by assuming that each permeant ion must traverse a series of energy barriers as it moves through the channel pore. Between each pair of adjacent barriers is an energy minimum or well, occupancy of which is energetically relatively favorable. In general, according to such a so-called "rate theory" model, highly permeant ions encounter low energy barriers and shallow wells, whereas ions that encounter one or more very high barriers are impermeant and those that enter very deep wells are minimally permeant and actually block the flow of permeant ions. Voltage-dependent sodium channels have been modeled in this way (4) using a three-well (site), four-barrier rate theory model, however, the number of barriers and sites and their positions werer chosen arbitrarily to allow for good fits to macroscopic current-voltage data. The basic outline of our modeling and the application of the model to the experimental data were described in report AN-1-1987.

Subsequent analysis revealed additional features. First using the optimal four-barrier, three-site model parameters, the occupancy by blocking calcium ion of each site was computed as a function of the membrane potential. The fraction blocked is proportional to the total occupancy. As can be seen from figure 1A below, where the occupancy of each site by calcium is indicated by the black vertical bar, external calcium can only enter the outermost two sites because the third (from the outside) barrier is too high to allow calcium to cross and permeate the channel. Hyperpolarization (more negative potentials) dramatically increases the channel occupancy (primarily at the central site) resulting in voltage-dependent block. In contrast, internal calcium (Figure 1B) can enter only the innermost site. Occupancy of this site is slightly favored by depolarization and thus block by internal calcium is only slightly voltage dependent.

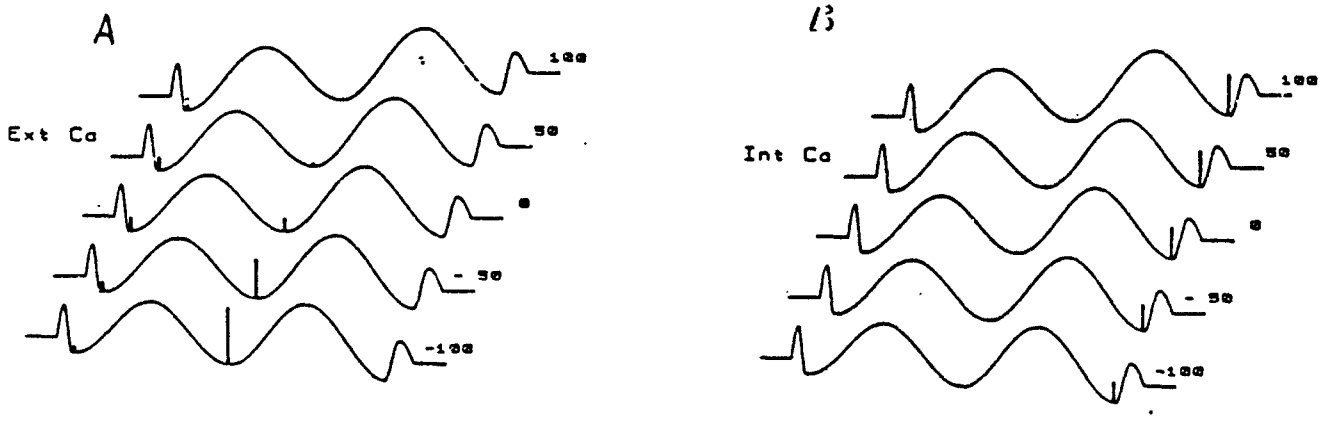


Figure 1. Calcium-occupancy in four-barrier, three-site model of the sodium channel in the presence of 125 mM Na^+. A. Probability of occupancy by Ca^{2+} vs V_m for 10 mM external Ca^{2+}. B. Same as A but internal Ca^{2+}.

plotted as a function of potential. Thus, at 10 mM external Ca^{2+}, the channel is much more occupied (blocked) at hyperpolarized potentials whereas at 10 mM internal calcium, the channel is nearly equally occupied (blocked) at all potentials. The fits of the model to the data are shown for external (Figure 3A) and internal (Figure 3B) calcium.

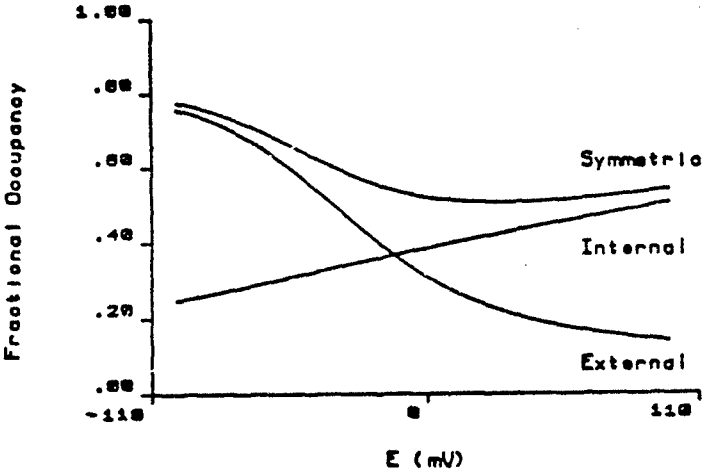


Figure 2. Total occupancy (related to block) by Ca^{2+} for rate theory model. Curves are shown for internal, external, and symmetrical, 10 mM Ca^{2+}

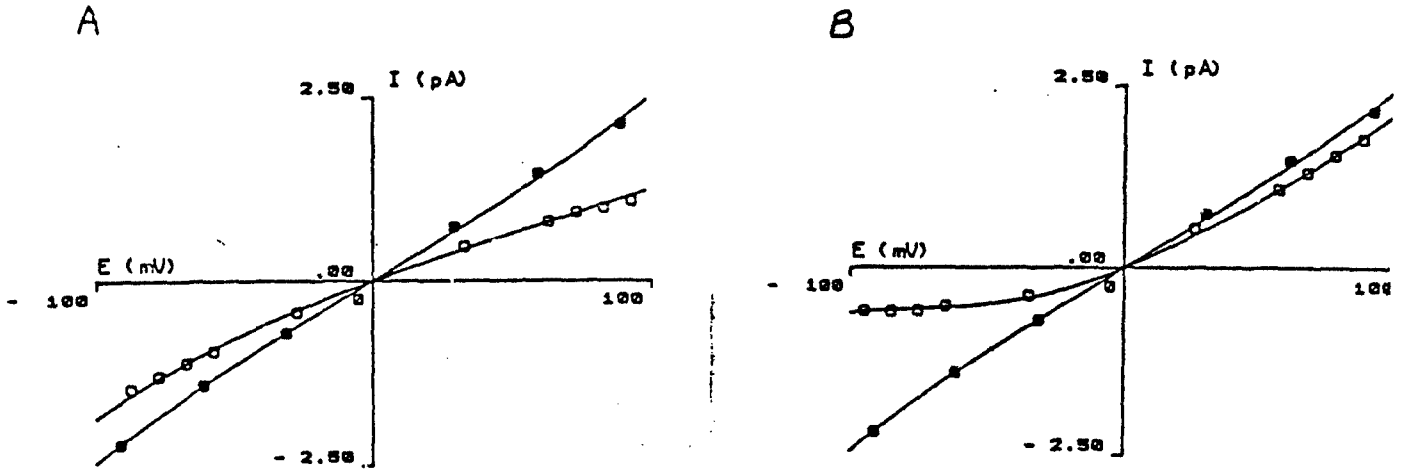


Figure 3. Fits of the model for symmetrical Na^{+} and internal (A) or external (B) 10 mM Ca^{2+}.

prediction is shown in Figure 4 for symmetrical 57 mM sodium with and without symmetricl 29 mM potassium. It can be seen that only outward currents are blocked by potassium. This voltage-dependent block has recently been experimentally verified independently by Garber and Miller (5) on muscle sodium channels in planar lipid bilayers.

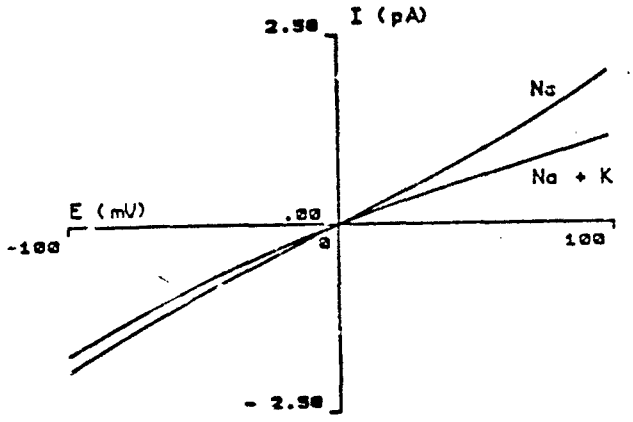


Figure 4. Rate theory model prediction of voltage-dependent block by symmetrical K^+.

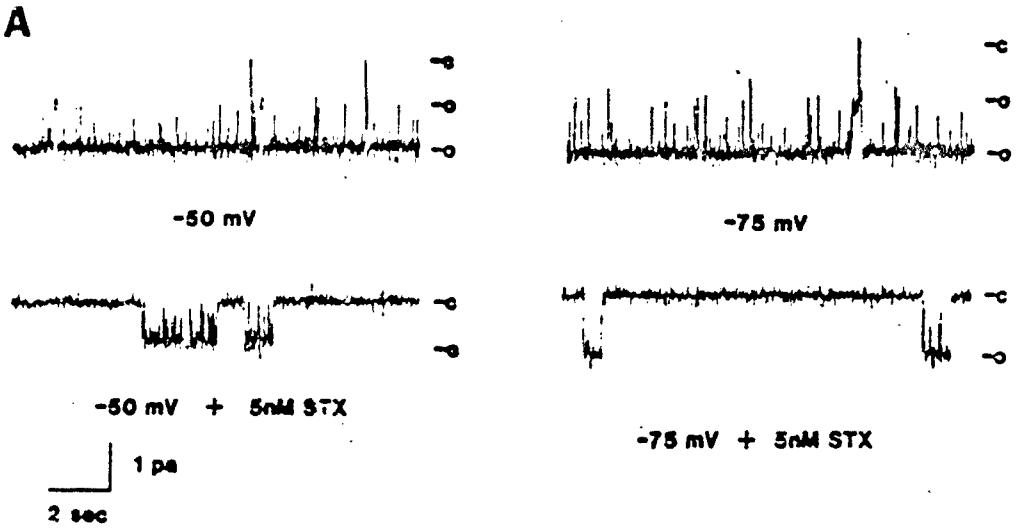
D. Purification and Characterization of Two Sodium Channels.

^{3}H-STX binding sites have been purified from homogenates of rat brain by solubilization in Triton X-100 and fractionation by normal and HPLC column chromatography (6). The binding sites were first fractionated by ion exchange and lectin-affinity chromatography. Concentrated samples were then sized by HPLC gel filtration and the final purification step was by HPLC DEAE ion exchange with a 100 - 250 mM KCl gradient. The overall purification scheme is summarized below:

SODIUM CHANNEL PURIFICATION

5-Step Method	Specific Activity (pmol/ml)	Yield (percent)	Purification (fold)
Solubilized P3	5.1	100	1
DEAE Sepharose	73	85	14
Hydroxylapatite	83	21	16
WGA	423	13	83
HPLC Molecular Sieve	1236	4.5	242
HPLC DEAE Gradient	2921	3.4	547

were incorporated into planar bilayers for determination of functional activity (2,7). In the presence of BTX, the channels were similar to unpurified channels incorporated directly from native membrane vesicles. Depolarizing potentials induced channel opening, the single channel conductance was about 25 pS in 150 mM NaCl and external STX blocked in a voltage-dependent manner (Figure 5).



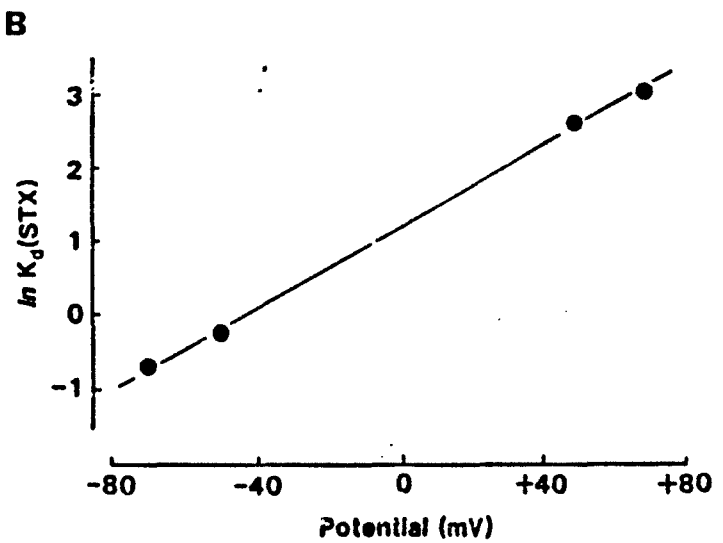


Figure 5. Voltage dependent block by STX. A Two purified BTX-activated channels were incorporated into a planar bilayer. Five nM STX induced long-lived closing (blocked) periods. B. The potency of block (inversely related to K_d) was greater at depolarized (positive) potentials.

In tracer flux experiments, veratridine (VER) has been found to act on sodium channels synergistically with polypeptide toxins from scorpion and sea anemone venoms (8). This synergism may be also be seen in bilayer experiments (ref. 9 and report AN-1-1986) in which polypeptide toxins increased the probability of the channel being open.

With purified channels, in the presence of VER, two STX-sensitive conductance states were observed (about 5 pS and 10 pS). Interestingly, sea anemone polypeptide neurotoxin dramatically increased the probability of being open for only the channels with the larger conductance (Figure 6). In preliminary experiments, critical examination of the polypeptide composition of active ^{3}H-STX binding fractions from the HPLC DEAE ion exchange column revealed that the early part of the peak was composed primarily of a 235kD α subunit whereas the later part of the activity peak contained a 265 kD α subunit. Scorpion polypeptide toxin greatly increased the open probability of the large conductance channel (235 kD, 10 pS) and increased the single channel conductance of the 265 kD channel from 5 to 9 pS. The possible relationship of these two types of brain sodium channels to the two distinct sodium channel genes reported by Noda et al. (10) will be investigated.

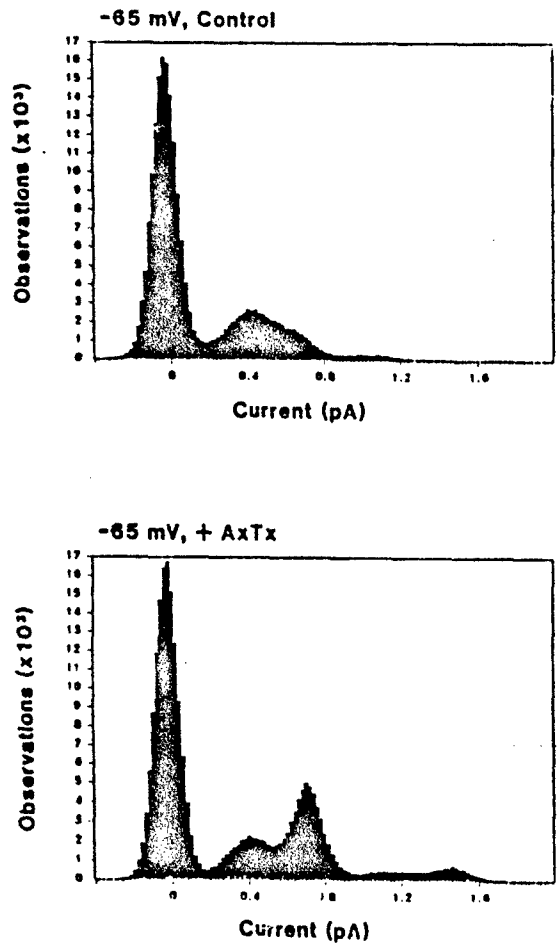


Figure 6. Amplitude histograms of purified, reconstituted sodium channels in planar bilayers in the presence of VER and sea anemone polypeptide toxin. Top: The large peak represents the closed state and the smaller peak is the combination of 5 and 10 pS sodium channels. Bottom: addition of 200 nM sea anemone toxin (AxTx) selectively increases the probability that the larger, 10 pS channel will be open.

E. Development of STX/TTX-sensitivity of Sodium Channels in Rat Cortical Astrocytes.

In collaboration with Dr. Paul J. Yarowsky, Department of Pharmacology and Experimental Therapeutics, University of Maryland, we have been studying changes in the STX and TTX sensitivity of sodium channels in neonatal rat cortical astrocytes. These glial cells, previous thought to have few if any voltage-dependent channels, have recently been shown to have a variety of channel types similar to those in nerve and muscle cells (11). In early cultures (up to about 7 days), there is substantial BTX-activated ^{22}Na-influx but virtually no detectable high affinity ^{3}H-STX binding activity. Block of influx by STX behaved as a single site blocking reaction with a K_i of more than 100 nM. From day 7 to 12, high-affinity (K_d = 1 nM) ^{3}H-STX binding activity increased rapidly with no overall change in BTX-activated influx. At two weeks or later, there were two components of block, one with a K_i estimated to be somewhat less than 1 nM and the other with a K_i of 100 - 500 nM. We interpret these results to indicate that initially, the astrocytes have only STX/TTX-insensitive sodium channels and that from 7 - 12 days in culture, some of these are replaced with (possibly newly synthesized) STX-sensitive channels that may be similar to those in neurons with a K_d of about 1 nM.

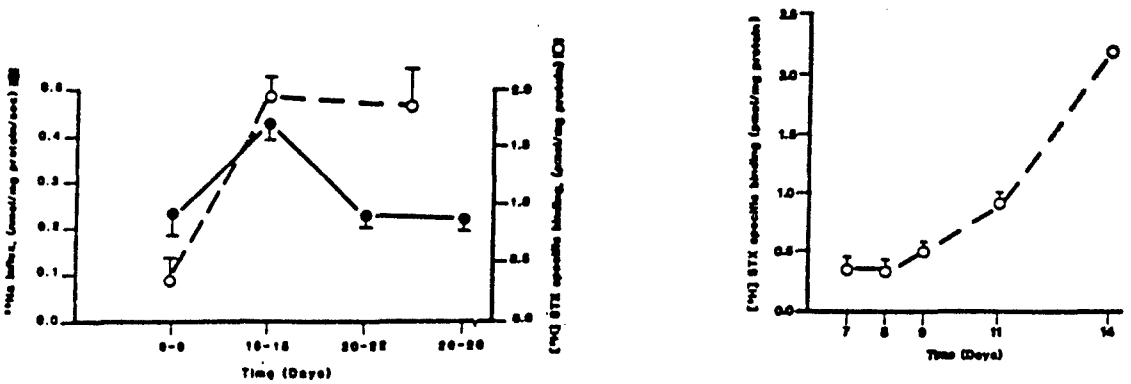


Figure 7. Changes in BTX-activated ^{22}Na influx (filled circles) and ^{3}H-STX binding (open circles) as a function of time in neonatal rat cortical astrocytes. Left: STX binding undergoes a five-fold change from day 6-8 to times older than 13 day. Sodium influx is about the same at early and later times. Right: detail of the increase in high affinity ^{3}H-STX binding from day 7 to 14.

F. Effects of Surface Charge on Sodium Channel Gating.

In experiments begun by Dr. S. Cukierman during the last month of this reporting period, we have found that both internal and external Ca^{2+} can shift the gating of BTX-activated sodium channel incorporated into planar lipid bilayers (11,12). Experiments were conducted with channels incorporated into either negatively-charged (phosphatidylserine) bilayers or neutral (phosphatidylethanolamine or phosphatidylcholine) bilayers. We believe that these effects are due to the interaction of Ca^{2+} with negative charges on both the lipids and the channel protein itself. Asymmetric binding of Ca^{2+} neutralizes the charge and alters the steepness of the transmembrane electric field sensed by the voltage-dependent gating machinery of the channel. We have found that 1. charges on the lipid headgroups and on the channel protein contribute about equally to these shifts, implying that the gating machinery is close enough to the charged lipid headgroups to be electrostatically affected, and 2. that there are more negative charges on the extracellular side of the channel than on the intracellular side.

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